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Award Number: DAMD17-99-1-9434

TITLE: Definition of the T Cell-Mediated Immune Response to Mammaglobin, a Novel Breast Cancer-Associated Protein

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REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Definition of the T Cell-Mediated Immune Response to Mammaglobin, a Novel Breast Cancer-Associated Protein

5. FUNDING NUMBERS
DAMD17-99-1-9434

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The elucidation of the immune response to cancer should be of great help in the development of new therapeutic strategies for the treatment of breast cancer. Based on recent advances in our understanding of antigen recognition by T lymphocytes, it has been possible to identify several tumor-associated antigens (TAA) recognized by CTLs. However, the expression for these TAAs has been shown to be relatively low in breast cancer tumor cells. A new protein named mammaglobin has been demonstrated to be exclusively expressed in the mammary epithelium. In addition, 90% of primary breast cancer tumors have high levels of expression of the mammaglobin protein. Given the exclusive mammaglobin expression in breast cancer tumors, this novel protein may prove to be a TAA highly specific for breast cancer that could be utilized in the near future for *in vitro* breast cancer-specific activation of CTLs. The discovery of mammaglobin-derived antigenic peptides that are highly expressed in breast cancer tumor tissue and are recognized by CTLs offer many exciting future therapeutic options for the treatment of breast cancer.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

A novel breast cancer (BC)-specific protein, mammaglobin, has been identified in our laboratories. Analyses of 15 human adult and 3 fetal tissues have demonstrated that mammaglobin is exclusively expressed in the mammary epithelium during proliferation and terminal differentiation. Significantly, high expression of mammaglobin has been found in 50% of human BC cell lines as well as in 62% metastatic primary BC tumors. Therefore, the characterization of the immune response to a specific and highly expressed BC-associated protein, such as mammoglobin should be of great importance toward the development of new immuno-therapeutic strategies for the treatment and prevention of this disease.

Body

Task 1. To determine whether mammaglobin-reactive T cells generated in vitro have the ability to lyse BC tumor cells.

We have obtained 15 different BC cell lines. Seven of these BC cell lines were found to be positive for mammaglobin expression. After HLA typing, three of the mammaglobin-positive BC cell lines were found to be HLA-A*0101, two were found to be HLA-A*0301, and one was HLA-A*0201. We developed lymphoblastoid cell lines (LCL) from peripheral blood mononuclear cells (PBMC) from 3 healthy male individuals that expressed matching HLA-A molecules to at least one of the BC cell lines mentioned above: No. 1: HLA-A*0101, No. 2: HLA-A*0301, and No. 3: HLA-A*0201. Subsequently, The LCL from each individual was transfected with the mammaglobin gene and then used for the development of mammaglobin reactive CD8+ T cell lines in vitro. The cytotoxic activity of these cell lines was tested using mammaglobin-transfected LCLs as well as one of several of the HLA-A matched BC cell lines mentioned above. All the original CD8+ T cell lines generated in vitro by this method showed high levels of cytolytic activity against both parental and mammoglobin-transfected LCLs but not against HLA-A-matched BC cell lines. Cytolytic activity against other HLA-A-matched LCLs but not BC cell lines led us to conclude that the CTL activity is not directed against mammoglobin but most likely against some EBV antigens.

Based on the results presented above, we developed a CD8+ T cell line from individual No. 3 against the mutant T2 cell line (HLA-A*0201) pulsed with a mammoglobin-derived peptide (aa 83-92) that binds the HLA-A*0201 molecule. We also developed CD8+ T cell lines from individual No. 2 against a HLA-A*0301-transfected mutant T2 cell line pulsed with mammoglobin-derived peptides (aa 23-31 or aa 31-39) that bind the HLA-A*0301 molecule. After 3 weekly stimulations, we tested the CTL activity of these CD8+ T cell lines against T2 cells loaded with the corresponding peptide and against an HLA-A-matched BC cell line. No mammaglobin- or BC-specific CTL activity was detected in any of the CD8+ T cell lines tested even in the presence of high levels of IL-2 (100 units/ml for 7 days). The mammaglobin-specificity of one of these cell lines was determined by means of the peptide-specific induction of expression of the CD69 activation marker as shown in Figure 1 (see appendix). Interestingly, mammaglobin-specific T cell lines from 2 healthy female HLA-A*0201-positive individuals using the same protocol could not be maintained in vitro for more that 1 week of culture. Further, we have developed mammaglobin-specific CD4+ T cell lines from three healthy individuals by means of incubating PBMCs in the presence of recombinant mammaglobin protein. As shown in Figure 2 (see appendix), one of the cell lines developed from a healthy male individual displays a mammaglobin-specific proliferative response. The same CD4+ T cell line did not show any cytolytic activity against a mammaglobin-pulsed self LCL. Interestingly, similarly developed CD4+ T cell lines from 2 healthy female individuals did not show any specific proliferative response against self antigen presenting cells pulsed with recombinant mammaglobin protein.These results strongly suggest that females may be tolerant to mammaglobin.

Task 2. To determine whether the BC-specific T cell immune response generated in vivo can recognize mammaglobin-derived antigenic peptides.

We developed CD8+ T cell lines from a HLA-A*0201-positive BC patient against self monocyte-derived dendritic cells (DC) pulsed with the mammoglobin-derived peptides 3-12 (SKM67) or 83-92 (SKM68). After 3 weekly stimulations, we tested the mammaglobin-specificity of both CD8+ T cell lines

by means of peptide-specific proliferative response against SKM67 and SKM68 peptides (Figures 3 and 4, respectively (see appendix)). In addition, the CTL activity of one of these CD8+ T cell lines (anti-SKM68) was tested against self DCs loaded with the corresponding peptide. A moderate mammaglobin-specific CTL activity was detected in this CD8+ T cell line tested as shown in figure 5 (see appendix).

Key Research Accomplishments

- ✦ Normal females are tolerant to mammaglobin.
- ✦ Breast cancer patients respond to mammaglobin, tolerance may be broken.

Reportable Outcomes

There have been no manuscripts, abstracts, presentations, patents and licenses applies for and/or issued, degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, informatics such as databases and animal models, etc., funding applied for based on work supported by this award, employment or research opportunities applied for and/or received on experiences/training supported by this award.

Conclusions

Overall, the data presented herein indicate that healthy female individuals have developed both CD4+ and CD8+ T cell tolerance to mammaglobin-derived peptides while healthy male individuals have not. Since mammaglobin is significantly over-expressed in BC tumors, the possibility exist that T cell tolerance may have been broken in vivo in these patients. Indeed, our positive results obtaining CD8+ T cell lines from a BC patient using peptide-pulsed DCs indicate this possibility. We have started limiting dilution analyses to determine the precursor frequency of mammaglobin-specific CD4+ as well as CD8+ T cells in both BC patients as well as healthy control female individuals in order to confirmed these results.

References

None used.

Appendices

Figure 1: Mammaglobin-specific activation of CD8+ T cells
Figure 2: Specific CD4+ T cell proliferative response to mammaglobin
Figure 3: Antigen specific proliferative response of induced CTLs after stimulation with cognate peptide (HLA-A2)
Figure 4: Antigen specific proliferative response of induced CTLs after stimulation with cognate peptide (HLA-A2)
Figure 5: Induction of CTL responses by peptide pulsed DCs

Appendices

Figure 1: Mammaglobin-specific activation of CD8+ T cells

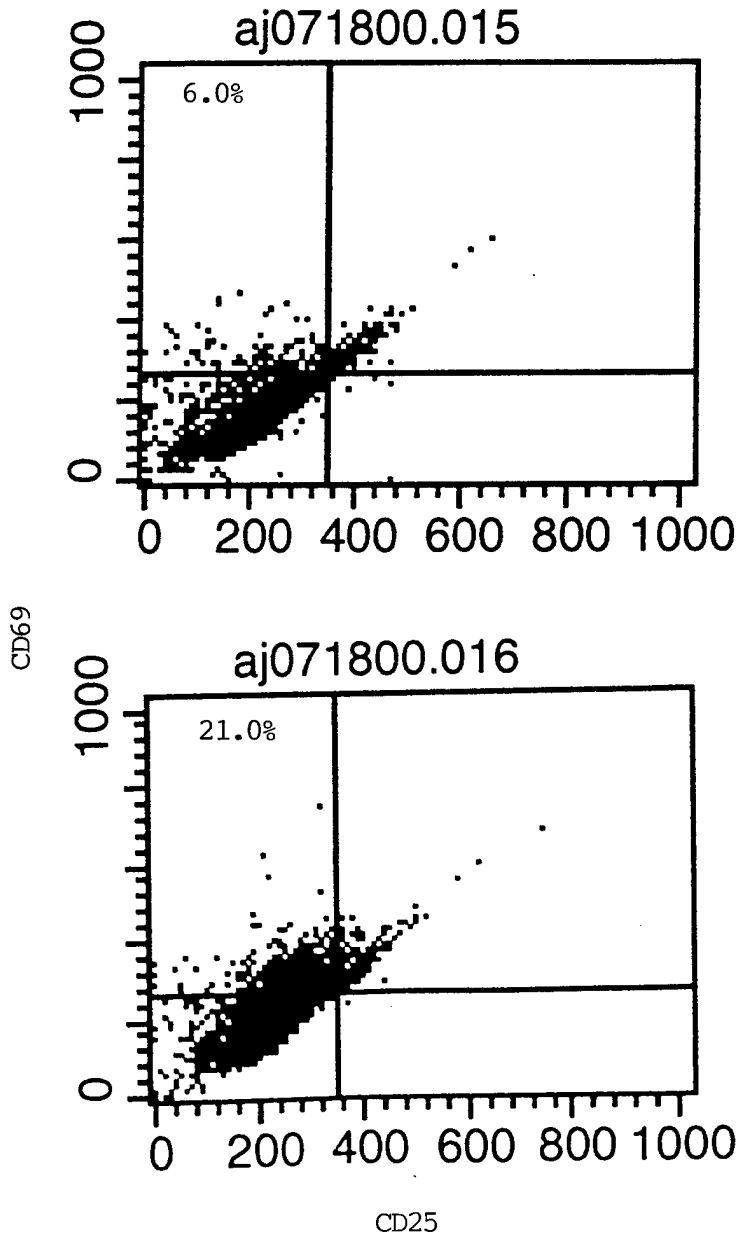
Figure 2: Specific CD4+ T cell proliferative response to mammaglobin

Figure 3: Antigen specific proliferative response of induced CTLs after stimulation with cognate peptide (HLA-A2)

Figure 4: Antigen specific proliferative response of induced CTLs after stimulation with cognate peptide (HLA-A2)

Figure 5: Induction of CTL responses by peptide pulsed DCs

Figure 1: Mammaglobin-specific Activation of CD8+ T cells



Stimulators

T_2 cells plus irrelevant HLA-A2-binding peptide

T_2 cells plus mammaglobin peptide 83-92

Figure 2.
Specific CD4+ T cell Proliferative Response to Recombinant Mammaglobin Protein

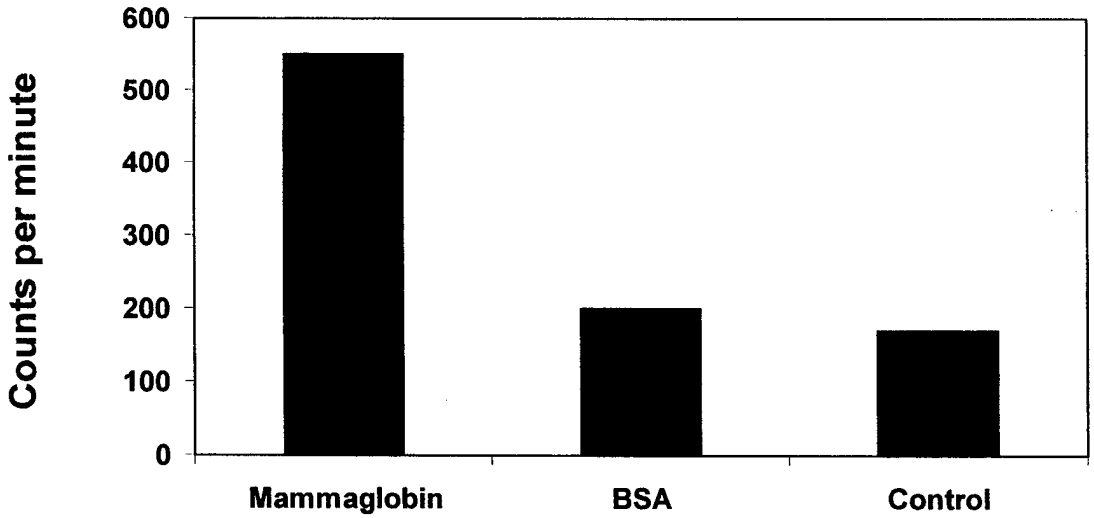


Figure 2. Specific CD4+ T cell proliferative response to recombinant mammaglobin protein. PBMCs from a healthy male individual were stimulated with 10 ug/ml of recombinant mammaglobin. On day 7, the CD4+ T cells were purified and re-stimulated in the presence of irradiated PBMCs and 30 U/ml of recombinant human IL-2. On day 14, the proliferative response to recombinant mammaglobin (5 ug/ml) was determine by means of ^{3}H-thymidine incorporation.BSA (5 ug/ml) or medium alone (control) were used a negative controls.

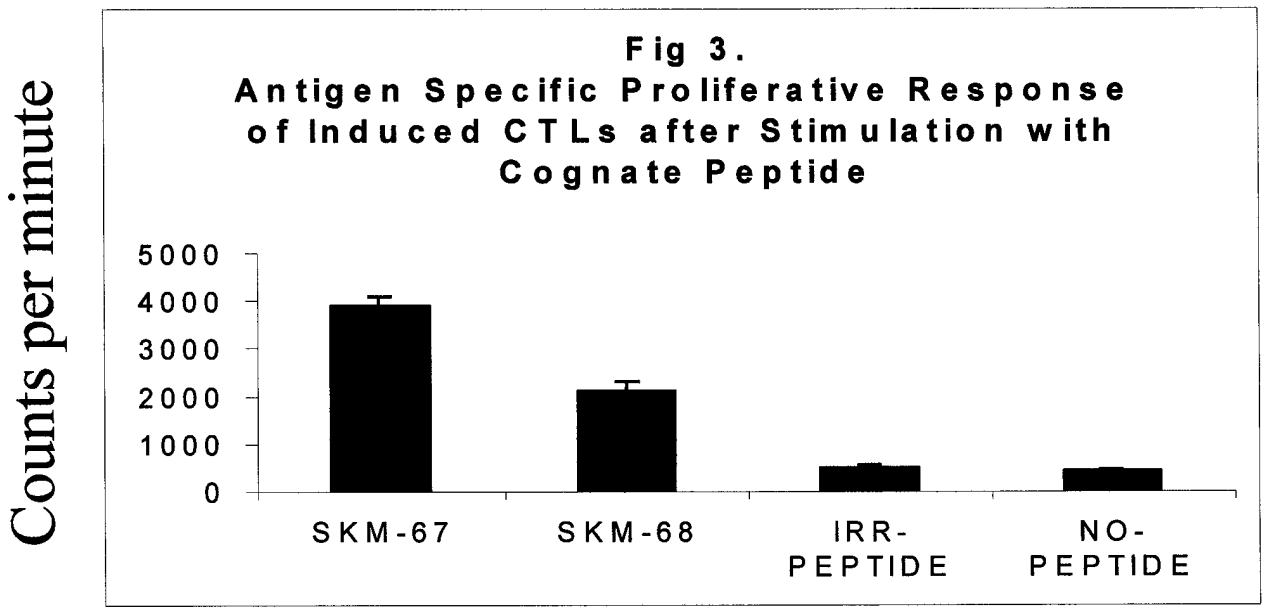


Figure 3. Ten days after the last stimulation with SKM-67(HLA-A2 peptide), triplicate wells containing $5x10^4$ cells from Ca-breast patient (HLA-A2) were cultured in presence of autologous irradiated DC ($5x10^3$) coated with the cognate peptide(SKM-67) or SKM-68, another mammoglobin specific HLA-A2 restricted peptide of similar anchor residue or irrelevant peptide(HLA-A3). 48 hours later, the cultures were pulsed with $^1H_3$ Thymidine, and harvested after an additional 16 hours. The assay was performed after 6 stimulation .

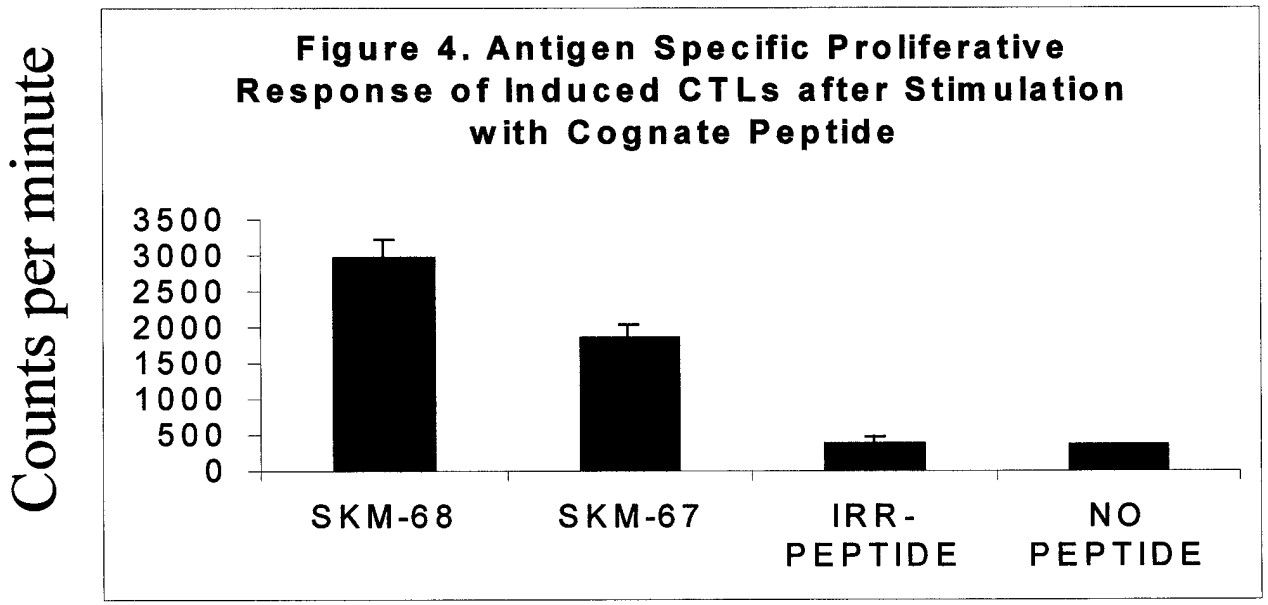


Figure 4. Ten days after the last stimulation with SKM-68(HLA-A2 peptide), triplicate wells containing $5x10^4$ cells from Ca-breast patient (HLA-A2) were cultured in presence of autologous irradiated DC ($5x10^3$) coated with the cognate peptide(SKM-68) or SKM-67, another mammoglobin specific HLA-A2 restricted peptide of similar anchor residue or irrelevant peptide(HLA-A3). 48 hours later,the cultures were pulsed with $^1H_3$ Thymidine, and harvested after an additional 16 hours. The assay was performed after 6 stimulation .

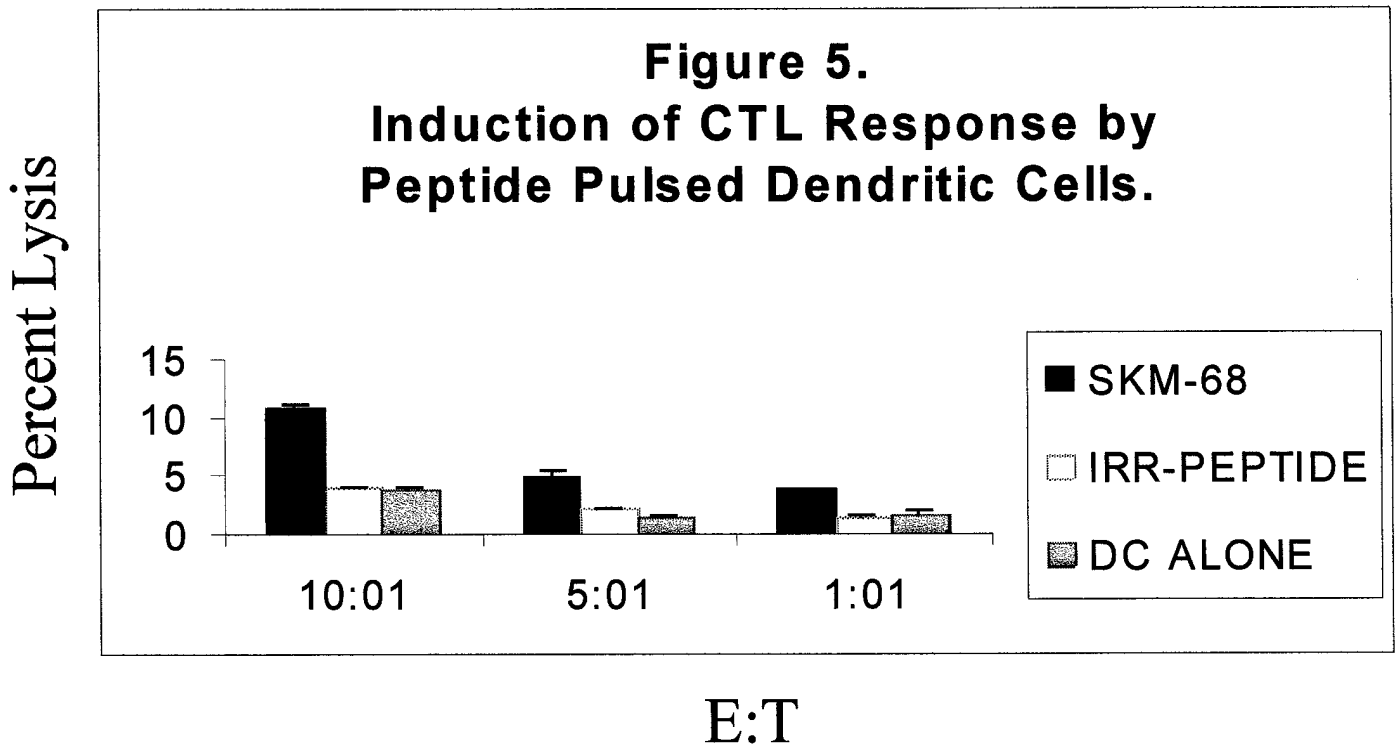


Figure 3. Adherent peripheral blood mononuclear cells were grown for 7 days in RPMI-1640 supplemented with GM-CSF and IL-4. DC pulsed with synthetic peptide derived from mammoglobin were used to induce a CTL response *in vitro*. Cytotoxic activity of induced CTLs was determined in a standard 4h ^{51}Cr release assay using autologous DC as target pulsed for 2h with 50 μg of the cognate peptide .